A Flexible, Robust Microbead-based Assay for Quantification and Normalization of Target Protein Concentrations

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Abstract

There are many methods for quantifying the concentration of a specific (target) protein in a sample, but current techniques are technically challenging or do not easily lend themselves to normalizing measured protein concentrations against the total amount of protein in the sample. Here, we describe a microbead-based assay for quantifying specific protein concentration(s) that is high-throughput, inexpensive, relatively simple to carry out, and which intrinsically incorporates normalization against the total protein content in each sample. This assay, which we term the FRANC assay, exploits high affinity biotin-streptavidin binding to couple sample proteins to streptavidin-labeled magnetic microbeads. Proteins so attached are then probed with one or more antibodies, followed by labeling of all proteins on the microbead with a fluorescent dye, and flow cytometry-based analysis. The FRANC assay demonstrates detection limits for target proteins in the single femtogram range, with an operating range up to as much as 10 nanograms of target protein. When quantifying total protein amount, the variation between different protein samples was similar to that of the bicinchoninic acid (BCA) assay. Finally, normalization of target protein concentrations resulted in at least an 80% reduction in variability as compared to non-normalized measurements. We conclude that the FRANC assay offers attractive advantages over current methods of quantifying specific protein(s) in complex samples.

Introduction

Quantifying the concentration of specific (target) proteins in a complex sample is an essential analytical technique for many biomedical applications. Samples typically consist of lysed tissue or other complex mixtures of multiple proteins[1], and current quantification methods employ antibody-based labelling and fluorescent, luminescent, or colorimetric determination of protein amounts[2,3]. For example, western blotting is commonly used for protein quantification[4,5]; it has the advantage of size separation by molecular weight, but the associated protein transfer can be technically challenging and slow [2,6,7]. Alternatively, enzyme-linked immunosorbent assays (ELISA), fluorescent microbead arrays, and related multiplex assays are now commonly used, as they allow for higher throughput [8-10]. The disadvantage of such assays, however, is limited flexibility, since kits containing validated antibody pairs for each protein of interest are often required.

An important consideration in all analytical techniques is sample normalization, i.e. the reporting of the concentration of the specific protein of interest in relation to levels of one or more reference proteins. Normalization accounts for user errors such as pipetting, as well as sample-to-sample variability, and is routinely performed in Western blot analysis by normalizing to a single reference protein (e.g. GAPDH, β-actin)[11-13]. While single reference proteins are sufficient for normalization in certain applications, reference protein amounts often vary between samples, making a single reference protein inadequate for sample normalization in many situations[14,15]. As a result, total protein-based normalization has been incorporated into Western blot analysis using different fluorescent or colorimetric total protein stains[16-19]. ELISAs and multiplex assays, however, are not well suited to total protein-based normalization.

Here, we describe a microbead-based platform to quantify the concentration of one or more specific (target) proteins within a sample. It combines the beneficial features of multiplex

assays with robust normalization against the total concentration of protein within each assayed sample. We refer to this as the FRANC assay (Flexible, Robust Assay for quantification and Normalization of target protein Concentration). We first describe the technology and then characterize the performance of the FRANC assay.

Results

Overview of FRANC Assay

The assay proceeds in four main steps. First, all proteins in a sample are attached to the surface of a microbead (Figure 1). Second, the microbeads are exposed to one or more fluorescently tagged antibodies to detect target proteins of interest. Third, carbodiimide crosslinking is used to conjugate fluorescent dye to carboxylic acid groups in all proteins on the surface of the microbeads. Finally, the microbeads are analyzed by flow cytometry to detect both the fluorescent signal from bound antibodies and the dye signal from all labelled proteins. Signal normalization can then be performed by expressing the antibody-specific signal as a ratio relative to the signal from all labelled proteins. For ease of sample handling, magnetic microbeads are used in the FRANC assay, since they can be quickly removed from solutions without sample loss using a 96-well magnetic plate (see verification in Supplementary Figure 1).

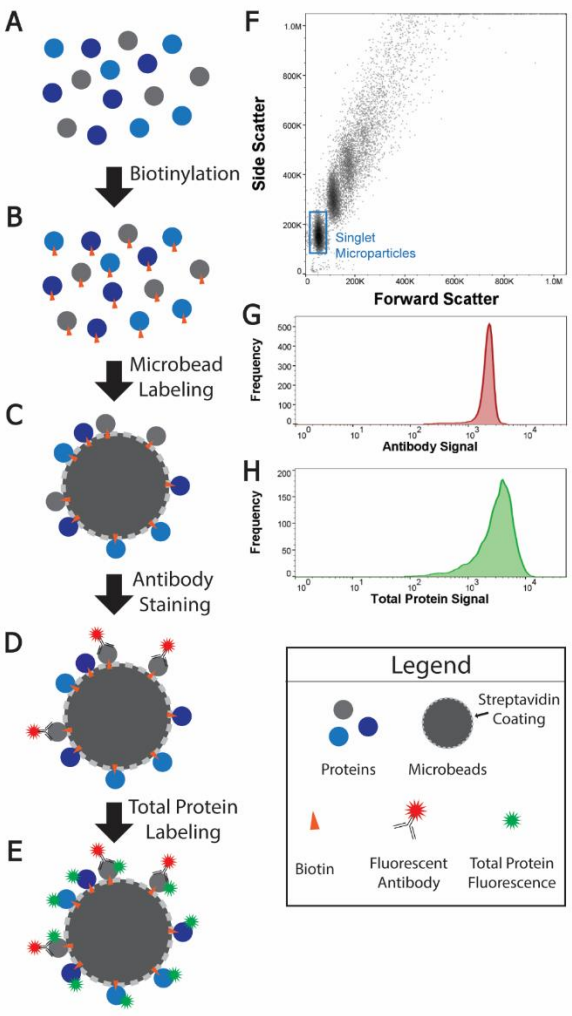


Figure 1. Schematic Overview of the FRANC Assay Process. (A) Protein samples are **(B)** biotinylated and **(C)** conjugated to magnetic microbeads through biotin-streptavidin interactions. **(D)** Microbeads are then exposed to fluorescently-labelled antibodies to tag specific proteins of interest, and **(E)** fluorescently labeled to determine total protein amount, which is required to normalize against protein amount. **(F-H)** Microbeads are analyzed by flow cytometry. **(F)** Forward vs. side scatter plots are used to distinguish singlet microbeads from aggregates. Both **(G)** antibody and **(H)** total protein signals are detected for each sample-conjugated microbead set.

Protein Biotinylation

There are several practical considerations that are important to the robust performance of the FRANC assay. For example, when attaching sample proteins to the microbead, it is appealing to simply allow proteins to adsorb onto the microbead surface, since this would permit

great flexibility in antibody labeling and subsequent steps. However, the affinity of such an attachment varies from one protein to another, so that a simple adsorption strategy would result in a biased protein sample on the microbead surface. To avoid this problem, we used biotin-streptavidin binding to conjugate proteins to the microbeads, since the high affinity of the biotin-streptavidin bond overcomes protein-to-protein differences in passive adsorption. This required that protein samples be biotinylated, and that microbeads be coated with streptavidin prior to exposure to the sample.

We biotinylated protein samples by using sulfo-NHS-LC-Biotin to modify protein primary amine groups (Figure 2A). To characterize the performance of this process, a range of protein:biotin mass ratios was tested, and signal from antibody-based labeling was measured for each sample. We used rabbit serum as the protein sample and detected the amount of rabbit IgG in this sample using a suitable fluorescently conjugated antibody. The operating range (defined as the linear range [$R^2 > 0.9$] of logarithmically-transformed antibody signal vs. the corresponding logarithmically-transformed sample protein amounts; see methods) spanned 4 or more dilution steps for protein:biotin mass ratios above 32 (Figure 2B). On the other hand, protein:biotin mass ratios less than 32 did not demonstrate an acceptable operating range. In addition, the dynamic output ranges (DOR, defined as the ratio between maximum antibody signal across the operating range to antibody signal for controls [no protein added]; see methods) were similar for protein:biotin mass ratios from 32 to 512, but were lower for a protein:biotin mass ratio of 2048 (Figure 2C). Given that protein:biotin mass ratios between 32 and 512 produced wide operating ranges and similar DORs, a protein:biotin mass ratio of 32 was selected for future testing since it most closely matched previously-used protein:biotin mass ratios and required less sample protein[20].

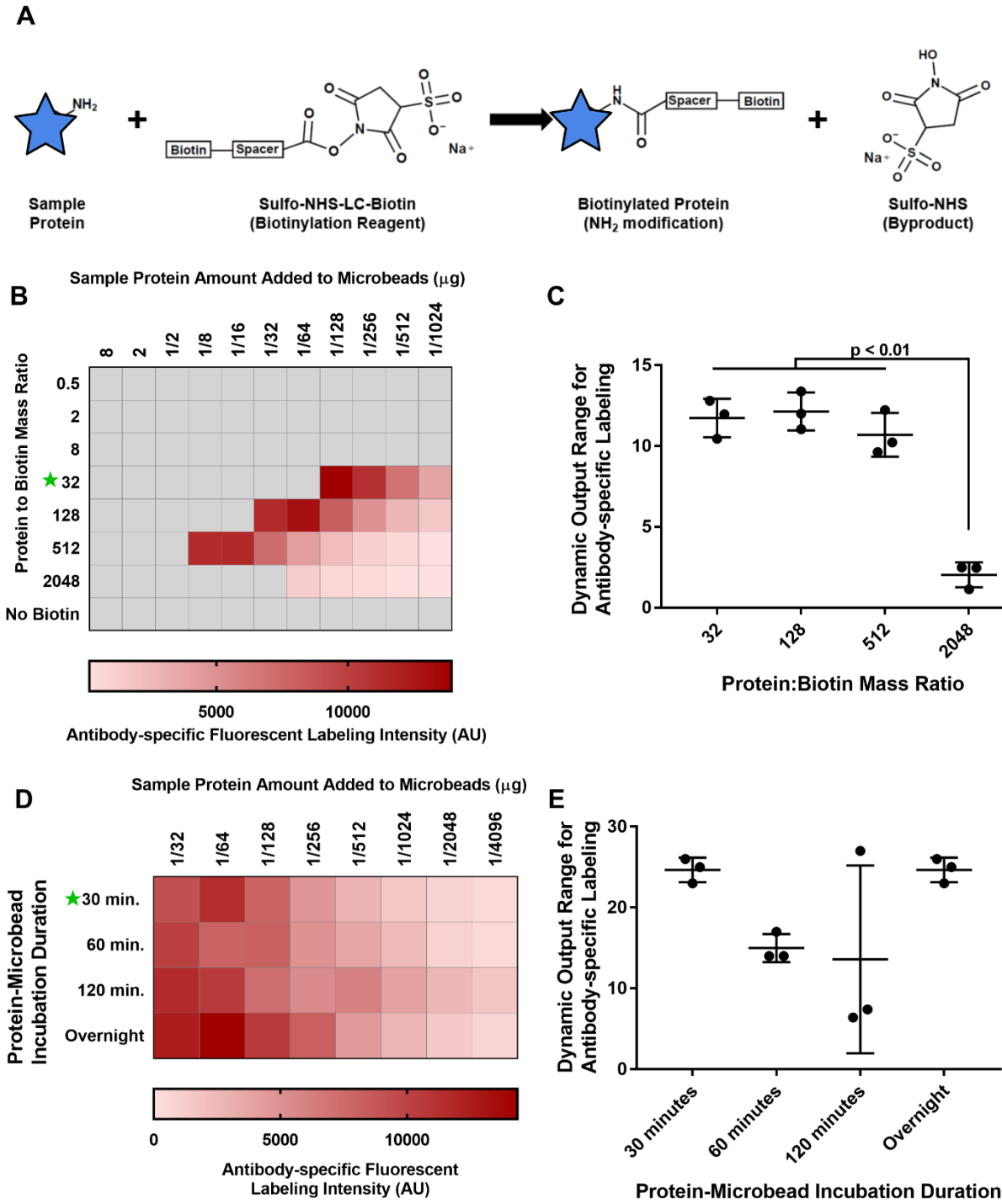


Figure 2. Effect of biotin labeling and protein-microbead incubation time on antibody labeling. **(A)** Overview of biotinylation of sample protein with sulfo-NHS-LC biotin through primary amine group modification[21]. **(B)** Effect of protein:biotin mass ratio on antibody labeling of IgG in a sample of rabbit serum. The heat map provides a pictorial representation of how labeling intensity varies with protein amount for each protein:biotin mass ratio, across the

assay's operating range ($R^2 > 0.9$; see methods). Conditions selected for the FRANC assay are denoted by the green star. Grey squares indicate conditions falling outside the assay's operating range. **(C)** The dynamic output range (DOR, see methods) vs. protein:biotin mass ratio. **(D)** Effect of protein-microbead incubation duration on antibody labeling of IgG in a sample of rabbit serum. The heat map provides a representation of how labeling intensity varies with protein amount for 30, 60, 120 minutes and overnight incubations of rabbit serum with microbeads. **(E)** The dynamic output range vs. protein-microbead incubation duration. For panels A and C, error bars denote standard deviation. n = 3 technical replicates for each dilution step. Statistical significance ($p < 0.05$) as indicated was determined by one-way ANOVA, post-hoc Tukey analysis.

Protein-Microbead Incubation Time

Next, we determined how assay results were affected by the duration over which biotinylated proteins were incubated with streptavidin-coated magnetic microbeads. Using manufacturer's recommendations, we considered incubation durations from 30 minutes to overnight. Antibody labeling of rabbit IgG in a sample of rabbit serum showed similar operating ranges across all tested incubation durations (Figure 2D). DORs were calculated for each incubation duration, with 30 minutes and overnight showing slightly higher ranges than other incubation durations (Figure 2E). However, differences between incubation durations were minimal, suggesting there is little advantage to extending incubation beyond 30 minutes. We thus selected a 30-minute protein-microbead incubation duration for future tests.

Antibody-based Fluorescent Labeling

Parameters for antibody-based labeling were next assessed, again using an antibody against rabbit IgG in a rabbit serum sample. The first parameter considered was antibody concentration: we tested concentrations ranging from 10ug/mL (a dilution factor [DF] of 50 for this antibody) to as little as 5ng/mL (DF of 10240). As expected, more antibody resulted in stronger signal; however, the extent of the operating ranges for antibody-labeling signal vs. sample protein amount were similar for each tested concentration (Figure 3A). Differences were more evident when assessing DORs (Figure 3B). At low antibody concentrations (2000 DF and

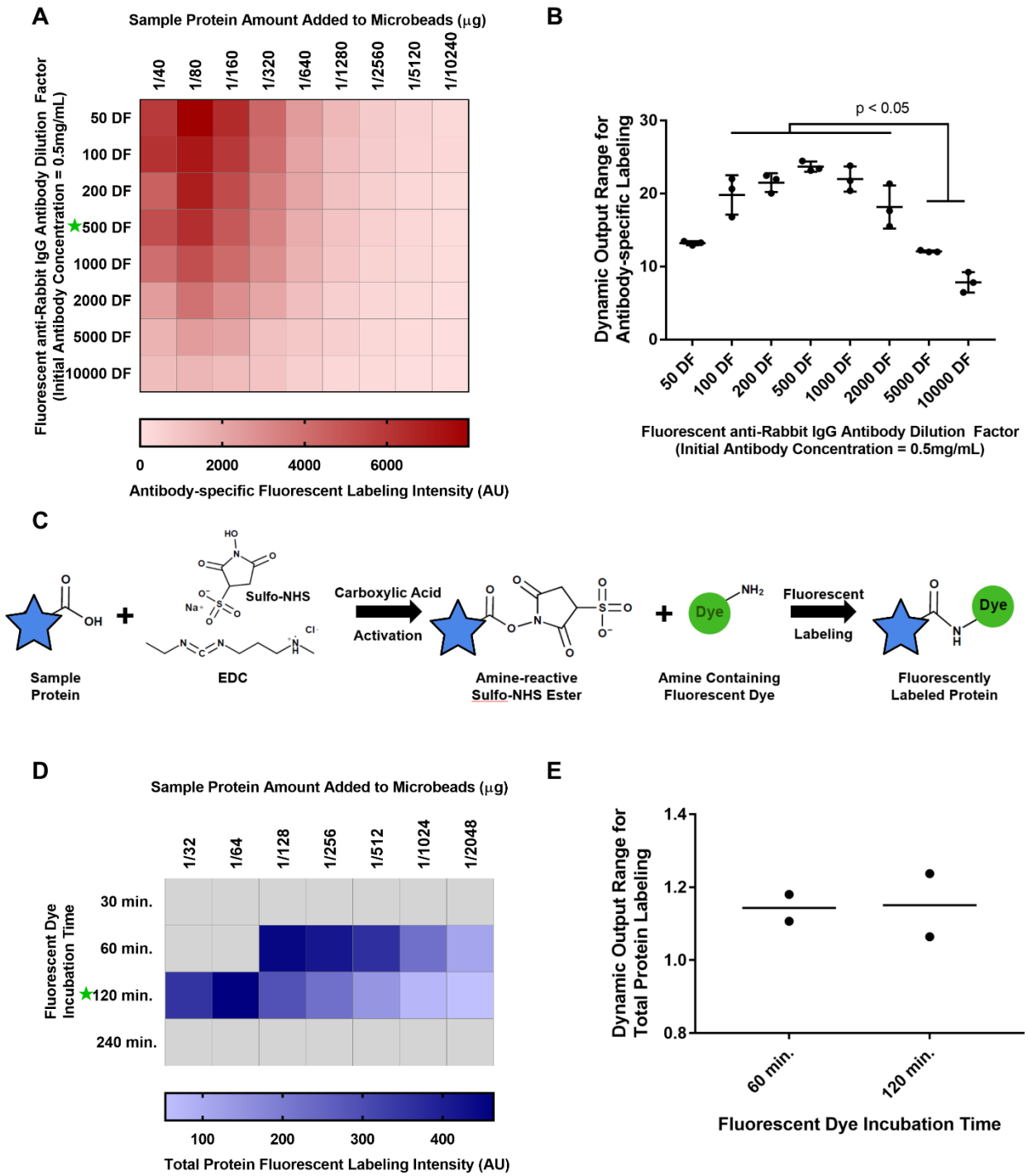
higher) the DOR was poor, while at high antibody concentrations (100 DF and 50 DF), the DOR was also poor due to increased background signal in controls with no sample protein. Intermediate antibody concentrations of 200, 500, and 1000 DF yielded similar DORs. Due to the sharp decline in signal beyond the 1000-fold DF, we selected a 500-fold antibody dilution factor (antibody concentration of 1 µg/mL) for subsequent testing.

Another important parameter in antibody labeling is the duration of the antibody incubation with sample protein on microbeads. The antibody concentration experiments described above were performed using 30 minute incubation durations. Additional experiments, using a 500-fold antibody dilution factor (1 µg/mL for this antibody), were performed for 60 and 120 minute incubations. Overall, the operating range was similar for all incubation durations (Supplementary Figure 2A) and the DOR was minimally impacted by incubation duration (Supplementary Figure 2B). As a result, we chose to continue using 30 minutes as the preferred duration for antibody incubation.

Total Protein Fluorescent Labeling

A key aspect of the FRANC assay is the ability to measure the total amount of protein conjugated to microbeads, and thus we investigated a number of parameters for their impact on the fluorescent labelling of total protein on beads. Carbodiimide-based conjugation of protein with dye was performed in two steps: (1) carboxylic acid activation by EDC and sulfo-NHS, and (2) fluorescent labeling with an amine reactive dye (Figure 3C). Thus, we first assessed different EDC concentrations, sulfo-NHS concentrations, and conjugation durations to determine how total protein signal depended on these parameters. Baseline parameter values, as determined from previous literature[20], were found to work well and were selected as the preferred conditions for the FRANC assay (Supplementary Figure 3).

For the second step, two primary amine-containing dyes were tested for total protein labeling: Alexa Fluor 488 Hydrazide and 4'(aminomethyl)fluorescein (AMF). Initial experiments utilized the hydrazide dye, but AMF was more cost-effective, particularly at higher dye concentrations. One hydrazide concentration (5 µg/ml) and 3 AMF concentrations (5 µg/ml, 50 µg/ml and 500 µg/ml) were tested immediately following carbodiimide exposure. We found that using AMF at concentrations of 50 and 500 µg/ml resulted in similar operating ranges for total protein fluorescent signal (Supplementary Figure 4A). While there was a greater DOR using 500 µg/ml of AMF, the results were more variable (Supplementary Figure 4B). Given the higher variability and cost associated with using 500 µg/ml of AMF, 50 µg/ml of AMF was selected as the optimal dye type and concentration. Fluorescent dye incubation duration was next assessed. Initially, a 120 minute incubation time was used, but 30, 60 and 240 minutes were also assessed using 50 µg/ml of AMF dye. The widest operating range was evident for 120 minutes, while 30 and 240 minutes did not result in a suitable operating range across tested sample protein amounts (Figure 3D). DOR range was similar for 60 and 120 minutes (Figure 3E). In view of its favorable operating range, 120 minutes was thus selected as the preferred incubation duration.



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181 **Figure 3. Effect of antibody concentration and total protein fluorescent dye incubation**
182 **duration on FRANC assay performance. (A,B)** Microbeads bound to rabbit serum were
183 incubated with different antibody concentrations (antibody dilution factors from 50 to 10,000-fold
184 from a starting anti-rabbit IgG antibody concentration of 0.5 mg/mL). **(A)** Effect of antibody
185 concentration on antibody labeling of rabbit IgG in a sample of rabbit serum. The heat map

provides a pictorial representation of how labeling intensity varies with antibody dilution factor, across the determined operating range ($R^2 > 0.9$; see methods). Conditions selected for the FRANC assay are denoted by green star. **(B)** The dynamic output range (DOR, see methods) vs. antibody concentration. Error bars denote standard deviations (n=3 technical replicates). **(C)** Diagram of carbodiimide conjugation of carboxylic acid functional groups in protein with EDC and sulfo-NHS. **(D)** Effect of AMF dye (50µg/ml) incubation time (30, 60, 120, and 240 minutes) on total protein labeling of a rabbit serum sample. The heat map is interpreted as in panel A; we use blue color coding to indicate that this panel refers to total protein labeling rather than antibody-based labeling of a target protein. Grey squares in the heat map indicate conditions falling outside the operating range. **(E)** The dynamic output range vs. total protein dye incubation time (n=2 technical replicates). Statistical significance ($p < 0.05$) was determined by one-way ANOVA, post-hoc Tukey analysis.

Analytical Sensitivity of the Assay

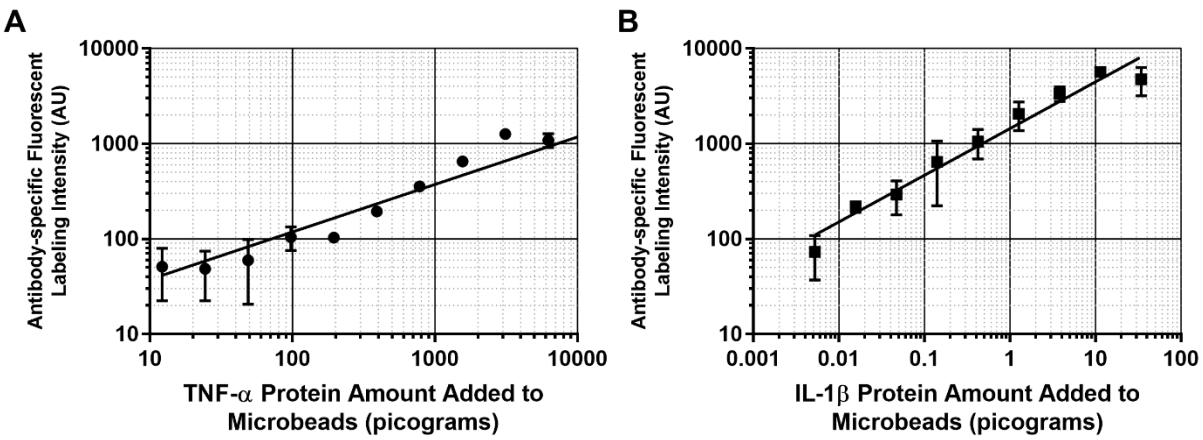
After determining suitable values for key parameters of the FRANC assay, overall assay performance was characterized, beginning with the assay's analytical sensitivity. We first carried out tests with two different recombinant proteins having different antibody labeling chemistry. Specifically, recombinant TNF-α was biotinylated and incubated with microbeads and was then detected with an anti-TNF-α antibody conjugated to a fluorophore (PE-Cy7). This resulted in an analytical sensitivity of 12 picograms of TNF-α protein and an operating range of 12 pg - 12.5 ng (Figure 4A). Additionally, recombinant IL-1β was detected with a non-fluorescent primary antibody and a fluorescent secondary antibody in two separate steps, producing an analytical sensitivity of 5 femtograms (fg) and an operating range of 5 fg to 34 pg (Figure 4B). The improved analytical sensitivity when using a secondary antibody is consistent with secondary labeling typically yielding more fluorophores per epitope.

Variability of total protein labeling for different protein samples

We next assessed how total protein labeling varied for different sample types, since it is imperative that the total protein labeling not be heavily impacted by protein sample composition. In testing the FRANC assay, it was necessary to quantify total protein concentrations in samples using an independent protein assay. For this purpose, we chose the widely-used BCA assay[22].

For this aspect of testing, we used six samples: three different sera (rabbit, goat, and mouse), primary mesenchymal stem cell (MSC) lysates, and cell lysates from untreated and from experimentally-treated HT-1080 immortalized cells. We plotted FRANC total protein signal (or BCA assay absorbance) vs. each sample's protein loading amount and computed the slope of each plot over the operating region. If the assays were insensitive to protein sample composition, all slopes would be identical; in practice, this is not the case and we therefore computed a coefficient of variation for the slopes over the six samples as a measure of the assay's sensitivity to sample composition. When using a linear regression methodology (conventional for the BCA assay; see methods) the coefficient of variation was lower with the FRANC assay (0.72 FRANC vs. 0.92 BCA). Comparing the slopes for the individual samples, 5 of the 6 samples had similar slopes for both assays, suggesting the FRANC assay performed similar to, or slightly better than, the BCA assay (Supplementary Figure 5A).

An alternative analysis approach is to log-transform the protein concentration and assay signal data (see methods) and regress this transformed data; doing so reduced the coefficient of variation for the slopes to 0.38 for the FRANC assay and 0.19 for the BCA assay. When comparing the log-transformed analysis approach to the more conventional direct approach, the FRANC assay performed similarly to the BCA assay for each sample (Supplementary Figure 5B). Interestingly, log-transformation reduced the coefficient of variation for the BCA method more than it did for the FRANC assay, likely due to the higher variability present at very low protein amounts in the FRANC assay's operating range (less than 0.1ng protein amount). These protein levels were not detectable with the BCA assay, which showed an analytical sensitivity of approximately 1µg. In conclusion, while variation between samples was apparent in determination of total protein levels for both the FRANC and BCA assays, the performance of these two assays was comparable, with slightly higher sample-to-sample variability in the FRANC assay being offset by greater sensitivity.



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241 **Figure 4. Determination of the analytical sensitivity (lower detection limit) for antibody**
242 **labeling.** Antibody-specific fluorescent label vs. protein amount for **(A)** TNF-α recombinant
243 protein detected by PE-Cy7 fluorescently-conjugated primary antibody and **(B)** IL-1β
244 recombinant protein with unlabeled primary antibody followed by PE fluorescently-conjugated
245 secondary antibody. The operating range is shown by a regression fit (solid line) to the log-
246 transformed data. Error bars denote standard deviations (n=3 technical replicates for each, in
247 some cases, error bars cannot be seen because the error is smaller than the data point).

248

249 *Total Protein Normalization*

250 Finally, we evaluated the ability of the FRANC assay to robustly normalize antibody
251 signal by the total protein signal. We detected rabbit IgG within a sample of rabbit serum using
252 AMF dye for simultaneous total protein labeling. As expected, the antibody and total protein
253 signals increased with sample protein amount (Figure 5A), with these two signals increasing at
254 comparable rates, as required for normalization. When the antibody signal was normalized by
255 the total protein signal, the ratio was relatively constant across sample protein amount, as
256 desired (Figure 5A). This behavior was quantified by the slope of plots of antibody signal (or
257 antibody signal normalized by total protein signal) vs. sample protein amount, where slope
258 values closer to zero suggest less antibody-labeling signal variability over the operating range. It
259 was observed that normalization reduced these slopes from 0.74 to 0.12 (Figure 5B). For
260 comparison, antibody signal was normalized to loaded rabbit serum sample protein amount, as

determined by the conventional BCA assay. This resulted in a slope of -0.25, which is further from the desired value of zero than the FRANC assay's total protein normalization methodology. In conclusion, the FRANC assay's total protein-based normalization was effective at accounting for loading variability and user error that cannot be accounted for when relying on protein quantification that is not fully integrated into the assay.

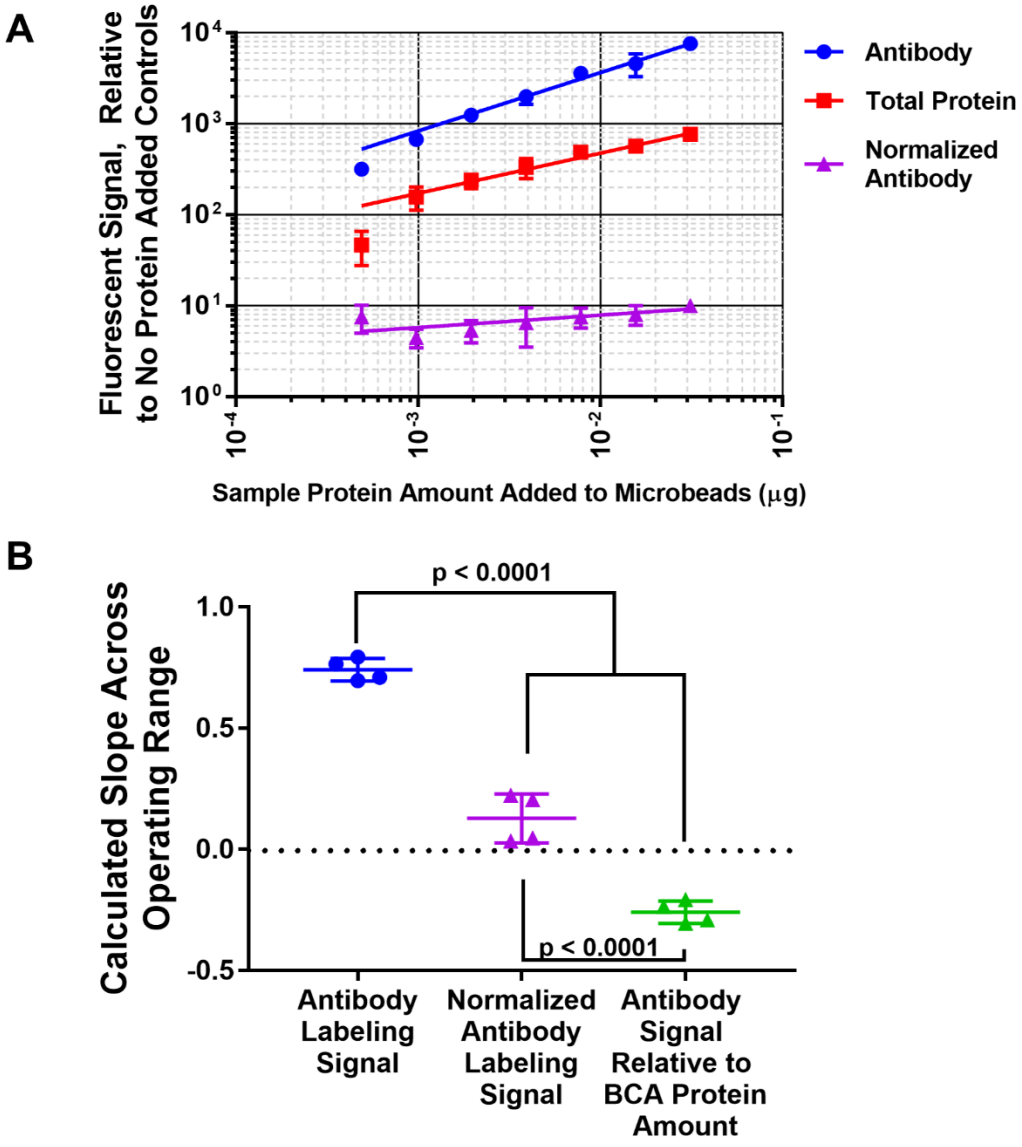

Figure 5. Normalization by total protein reduces assay variability. **(A)** A rabbit serum sample was probed with fluorescently-tagged anti-rabbit IgG, followed by measurement of total protein (n=4 technical replicates). Normalized antibody signals were calculated by dividing antibody signal by respective total protein signal across the operating range. Operating range for antibody, total protein, and normalized antibody are shown (solid regression lines) on a log-log plot of assay signal vs. sample protein amount. **(B)** Slope of regression to fluorescent signal vs. sample protein amount over the operating region for antibody signal, antibody signal normalized to total protein, and antibody signal relative to loaded sample protein amount, as determined by BCA assay (n=4 technical replicates). Error bars denote standard deviation. Statistical significance ($p < 0.05$) was determined by one-way ANOVA, post-hoc Tukey analysis.

Discussion

The FRANC assay we describe here provides a platform for quantification of specific (target) proteins within a complex mixture. The assay is capable of robustly accounting for sample variability through total protein normalization in a manner that is integrated with the overall assay. While western blotting can account for loading and sample variability with various normalization approaches, it is slow and technically challenging compared to other methods. Conversely, ELISA and multiplex assay-based protein quantification is much faster, but normalization is not intrinsically feasible within the assay, since only a subset of proteins in the sample (i.e. those recognized by “capture” antibodies on microbeads or well-plates) is assayed. Thus, there is a need for an improved method for quantifying specific (target) proteins within a sample. The FRANC assay we report here addresses these shortcomings by combining a microbead platform, which allows for high-throughput simple protein handling in multiplex assays, with total protein-based signal normalization.

A key aspect of the FRANC assay is the use of biotin-streptavidin interactions for sample protein conjugation to microbeads. By conjugating biotin to primary amine groups in a protein sample using sulfo-NHS chemistry, we can exploit the strong affinity between biotin and streptavidin, ensuring that all proteins in a sample bind to the microbead surface with similar affinities, regardless of protein structure. This key step overcomes a limitation of passive surface

adsorption [23-27], i.e. the differential affinity of different proteins to passively bind to microbeads. We tested a wide range of protein:biotin mass ratios and found that lower (i.e. more sensitive) protein detection limits were achievable when more biotin was added. However, this came at the cost of poorer antibody binding. This understanding allows assay flexibility depending on factors such as antibody affinity to the target protein, sample amounts, and the required assay sensitivity. Additionally, the biotinylation chemistry could be altered to bind other amino acid sequences **[20]** or even to bind only the N-terminus by modifying the incubation pH [28,29].

The FRANC assay is compatible with traditional antibody-based fluorescent labeling of specific proteins of interest. After protein conjugation to microbeads, antibody labeling occurred with an incubation time of as short as 30 minutes, and with antibody concentrations as low as 0.5 µg/mL. In addition, the FRANC assay is compatible with direct and indirect antibody labeling. While multiplex tests were not performed, the FRANC assay is also expected to be compatible with a wide range of fluorophores, including simultaneous multi-antibody labeling which is commonly performed with 10 or more probes in traditional flow cytometry [30]. Although not observed in this study, it is possible that some antibody-antigen interactions could be inhibited due to the biotinylation of primary amine groups; if this were suspected, reducing the degree of sample biotinylation would be recommended.

The final component of the FRANC assay is fluorescent labeling of the total protein attached to microbeads, essential for sample protein normalization. While single reference proteins are often used in western blot analysis for normalization, the relative concentrations of many reference proteins varies across experimental conditions[12-14]. Total protein labeling offers a more robust normalization method [17,18]. For the FRANC assay, total protein labeling was performed by conjugating primary amine-containing fluorescent dyes to carboxylic acid groups. After determining the preferred concentrations for crosslinkers and fluorescent dye, we observed that the operating range for total protein signal approximately coincided with the

operating range for antibody-specific labeling. Thanks to these similar operating ranges, normalization was possible by expressing antibody labeling relative to total protein labeling, which reduced the variability by approximately 6-fold. This is an extreme example to demonstrate the normalization capabilities of the FRANC assay, since practical assay applications would not use normalization over such a wide range of protein amounts, but would instead utilize normalization to account for loading error and slight quantification differences between replicates and sample types. This scenario was illustrated by the ability of the FRANC assay's normalization approach to reduce variability to about half the variability observed when using total protein amounts determined separately from the BCA assay. Thus, robust signal normalization enhances assay quality and is an important feature of the FRANC assay.

Total protein normalization is only useful if labeling of total protein content is consistent between different protein samples. We tested six different protein samples with the FRANC assay, and determined that the assay variability was very similar to that of the well-established BCA assay, which suggests that the total protein labeling approach is not heavily skewed by amino acid composition. However, this was true only when results were analyzed using traditional linear regression of assay signal vs. protein loading, commonly used with the BCA assay. Logarithmic transformation of the data decreased the variability of both assays, although the decrease was less impressive for the FRANC assay vs. the BCA assay, suggesting greater variability at lower total protein signals with the FRANC assay. If end-users required reduced variability for small protein amounts, different functional groups could be targeted, or brighter total protein fluorescent dyes utilized. Further improvements to total protein signal are possible by simultaneously combining multiple chemistries to detect multiple functional groups.

Table 1. Overview of the performance of the normalized protein expression assay

Specification	Normalized Protein Expression Microbead Assay
Normalization Capability	Strong. Intrinsic to each assay sample for accounting for sample loading and other user errors
Lower Detection Limit	TNF-α + PE-Cy7 fluorescent primary antibody: ~24 pg IL-1β + unlabeled primary antibody + PE secondary antibody: 0.2 fg
Linear Operating Range	TNF-α : ~12 pg to ~12.5 ng IL-1β: ~5 fg to ~34 pg
High-Throughput Aspects	Applicable to 96 or 384-well plate formats, multiplex fluorescent antibodies up to flow cytometer limitations
Required Protein Sample Amount	Less than 0.5 µg
Overall Assay Procedure Time	5 to 6 hours from protein sample to flow cytometric analysis
Hands-on Time	Approximately 1 hour
Reagent Cost per Sample	Approximately $3 per 12-step, single sample dilution curve

Overall, the FRANC assay was attractive compared to conventional western blotting, ELISA, and multiplex assays, as summarized in Table 1. Sensitivity was found to be 12 picograms protein for direct antibody labeling, which is comparable to ELISAs, multiplex assays, and conventional western blotting[31,32]. Indirect antibody labeling resulted in detection in femto- to picogram quantities, similar to recent advances in microfluidic western blotting that have also reported detection of femtogram quantities[33]. The FRANC assay requires approximately 5 to 6 hours from start to finish, of which most time is “hands-off” incubations, leading to approximately 1 hour of hands-on time. The cost of the required reagents and quantities needed per 96-well sample are modest, resulting in a cost of approximately $3 per 12-step, single sample dilution scheme. However, it is recommended that end-users confirm reagent concentrations and antibody dilutions for their specific application, which will affect the assay cost. In conclusion, the normalized protein expression microbead assay presented herein is an improved approach that integrates total protein labeling, important for assay normalization, with traditional fluorescent antibody-specific protein labeling.

Methods and Materials

Sample preparation and sourcing

A variety of protein samples, representing a range of situations encountered in laboratory work, was used for testing the FRANC assay. Goat (LAMPIRE Biological Laboratories, Pipersville, PA, USA), rabbit (Gibco, Waltham, MA, USA) and mouse (Invitrogen, Waltham, MA, USA) sera were obtained commercially. Primary adipose-derived mesenchymal stem cells (Lonza, Basel, Switzerland) were grown to confluence and were then lysed using RIPA buffer (Thermo-Fisher, Waltham, MA, USA) supplemented with Halt protease inhibitors (Thermo-Fisher, Waltham, MA, USA). Similarly, immortalized HT-1080 fibrosarcoma cells (American Type Culture Collection, Manassas, VA, USA) were lysed without treatment or after 24 hours of treatment with 25 ng/mL phorbol 12-myristate 13-acetate (PMA, MilliporeSigma, St. Louis, MO, USA)[34]. PMA is known to activate protein kinase C and alter downstream protein expression, such as matrix metalloproteinases[34,35]. Recombinant TNF-alpha and IL-1β in 0.1% (w/v) bovine serum albumin carrier protein (R&D Systems, Minneapolis, MN, USA) were reconstituted in phosphate buffered saline prior to use. The total amount of protein in each sample was quantified by the bicinchoninic acid assay (BCA, Thermo-Fisher, Waltham, MA, USA) per the manufacturer's protocol using a bovine serum albumin standard curve, and a BCA reagent incubation time of 30 minutes at 37^{o}C.

FRANC Assay Method

Sulfo-NHS-LC-Biotin (sulfosuccinimidyl-6-[biotin-amido]hexanoate, Thermo-Fisher, Waltham, MA, USA, Supplementary Figure 1) was incubated with 10 µg of protein sample at a mass ratio of 32:1 sample protein:Sulfo-NHS-LC-Biotin for 1 hour at room temperature. These

biotinylated samples were diluted as desired and loaded into 96 well plates (50µL sample per well). Streptavidin-coated hydrophobic magnetic microbeads (Dynabeads MyOne Streptavidin T1 [10mg/mL], Thermo-Fisher, Waltham, MA, USA) of 1.05 µm diameter were washed twice with 0.5% (v/v) Tween^{TM}-20 (Fisher Scientific, Waltham, MA, USA) phosphate buffered saline (referred to as T-PBS) by adding 1mL of T-PBS to 12µL of microbeads and subsequent microbead removal from solution using a neodymium bar magnet (0.75" length x 0.25" width x 0.25" height, 10.5 lbs pull force, K&J Magnetics, Pipersville, PA, USA). Microbeads were then resuspended in T-PBS at 20µg/mL (500 dilution factor) and 50 µL of bead suspension was added to each protein sample. Protein samples and microbeads were then allowed to incubate for 30 minutes at room temperature.

After incubation, microbeads were magnetically removed from suspension using a custom-made 96 well magnetic plate separator (Supplementary Figure 1, 96 ¼" diameter x ½" thick cylindrical magnets assembled in a 96-well plate, 4.8 lbs. pull strength for each magnet, K&J Magnetics, Pipersville, PA, USA). Specifically, the 96-well plate was placed on top of the magnetic separator for 30 seconds, after which the solution was removed from each well by inverting the plate over a waste reservoir. Microbeads were washed twice with 100µL of T-PBS and incubated with 50µL of the appropriate antibody solution for 30 minutes at room temperature, followed by washing with T-PBS.

To detect all proteins in the sample ("total protein") by carbodiimide crosslinking, 50µL of 1 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, MilliporeSigma, St. Louis, MO, USA, Supplementary Figure 6) and 2.5 mM N-hydroxysulfosuccinimide (sulfo-NHS, MilliporeSigma, St. Louis, MO, USA, Supplementary Figure 6) in T-PBS were added to microbead suspensions and immediately agitated and incubated for 15 minutes at room temperature. Following two T-PBS washes, 50 µL of 50 µg/mL 4'-(aminomethyl) fluorescein (AMF, Thermo-Fisher, Waltham, MA, USA, Supplementary Figure 1) dye in T-PBS was added

to the microbead suspension and incubated for 2 hours at room temperature under agitation. After 2 additional T-PBS washes, microbeads were resuspended in 200 µL T-PBS and analyzed by flow cytometry (Attune NxT, 12.5ul/min, 25µl volume, Thermo-Fisher, Waltham, MA, USA).

Confirming Magnetic Separation of Microbeads

To confirm that microbeads were not washed away when immobilized by the external magnet during wash steps, microbeads were counted by flow cytometry before any wash steps and after each of three wash steps. 50 µL of diluted microbeads (20µg/mL in T-PBS) were added to each well of a 96 well plate. Unwashed controls were immediately collected from the plate by pipetting and placed in 1.5 mL microtubes for subsequent flow cytometry analysis. The remaining microbead samples were washed three times with T-PBS, and samples were collected into 1.5 mL microtubes after each wash step.

Determining Suitable Assay Conditions

We extensively tested the effects of changing key assay parameters, e.g. concentrations of reagents and durations of key steps, in the FRANC assay. Starting (baseline) values for assay parameters and reagent concentrations were informed by protocols for related bioconjugation techniques[20]. For simplicity, rabbit serum was initially used as the protein sample, and we assayed the amount of rabbit immunoglobulin G (IgG) in the sample using a commercial fluorescent antibody (Donkey F[ab']2 Anti-Rabbit IgG – Phycoerythrin [PE], Abcam, Cambridge, United Kingdom)[9,36,37]. Below we describe which assay protocol parameters were assessed, the justifications for baseline parameter values, and how tests were conducted.

For each parameter, two key outputs were used to determine suitable assay conditions: the assay's operating range and its dynamic output range (DOR). The operating range was determined from plots of logarithmically transformed antibody-specific labeling (y-axis) vs.

logarithmically transformed amount of sample protein (x-axis), and was defined as the largest possible sample protein region (x-axis) over which: (1) y-values were greater than vehicle-only controls, and (2) coefficient of determination values (R^2) for linear regression of y on x were greater than 0.9. Log-log transformation was utilized to allow analysis of faint antibody signals and small protein loading amounts that are often under-weighted in traditional linear analysis. The DOR was defined as the maximum signal magnitude (y axis value) in the operating range divided by the signal (y axis value) for the vehicle-only controls.

In addition, we carried out a similar analysis using total protein fluorescent signal as the quantity plotted on the y-axis to determine the operating range and DOR for total protein labeling. We then sought assay conditions resulting in a large operating range and a large DOR for both antibody-specific labeling and total protein labeling, since this maximized the assay's ability to accurately detect quantitative differences between protein samples.

Levels of biotinylation

Higher levels of sample protein biotinylation may inhibit antibody-specific labeling, while lower biotinylation levels may lead to poor coupling of sample proteins to streptavidin-coated beads. Thus, it was important to determine a suitable level of biotinylation. Previous studies have suggested that molar biotin:protein ratios of less than 5:1 reduce protein aggregation, insolubility, and inhibition of antibody-labeling [20]. Furthermore, biotin:protein ratios as low as 2:1 have been shown to result in effective streptavidin binding [38]. However, the use of molar ratios is impractical when using complex samples containing a mixture of proteins. Instead, we used mass ratios; assuming an average protein size of 53 kDa[39], 2:1 and 5:1 biotin:protein molar ratios correspond to idealized protein:biotin mass ratios of approximately 45:1 or 18:1, respectively. The approximate mean of these two mass ratios, namely 32:1 protein:biotin, was

used as a starting point for further testing. Mass ratios greater than and less than 32:1 were assessed to determine their effect on labeling of specific proteins and on total protein labeling.

Rabbit serum was incubated with biotin for 1 hour at protein:biotin mass ratios from 2048:1 to 0.5:1, or without biotin. Biotinylated rabbit serum samples were then loaded into 96 well plates using the following serial dilution structure: 8 µg protein/well, 4-fold serial dilutions from 2 to 1/8 µg protein/well, and 2-fold serial dilutions from 1/16 to 1/1024 µg protein/well. Streptavidin-coated microbeads were then added to each well for 30 minutes to conjugate protein. Next, antibody-specific labeling was performed with a fluorescently (PE fluorophore) conjugated anti-IgG antibody. The operating range and DOR were determined for each protein:biotin mass ratio.

After determining the preferred protein:biotin mass ratio, biotin-protein incubation durations of 15, 30, 60, and 120 minutes were tested, using an approach as described above. The operating range and DOR were determined for each incubation time.

Protein-microbead incubation duration

Next, we investigated the effect of incubation duration for biotinylated proteins with microbeads, considering incubation durations from 30 minutes to overnight. For this purpose, we used rabbit serum samples, diluted into 96-well plates, detected with antibodies against IgG, as described above. The operating range and DOR were determined for each incubation time.

Antibody labeling

To optimize antibody labeling, we used rabbit serum protein as the sample and an anti-IgG antibody, as described above. We first considered the effect of antibody concentration, testing antibody concentrations from 10ug/mL (50 DF for the antibody) to as little as approximately 5ng/mL (10240 DF). We then examined the effect of antibody incubation

duration, considering durations from 30 minutes to 120 minutes. The operating range and DOR were determined for each incubation duration.

Total protein labeling

Fluorescent labeling of the total protein conjugated to microbeads in each sample is necessary for normalization as an integral part of the FRANC assay. Since primary amine groups were utilized for protein biotinylation, carboxylic acid residues, found in glutamic and aspartic acid amino acids as well as the protein C-terminus, were targeted for this purpose[39]. Specifically, a carbodiimide crosslinker was used to conjugate a primary amine-containing fluorescent dye to carboxylic acid residues (Figure 3C). To minimize hydrolysis and subsequent loss of reactivity of the unstable acylisourea intermediate formed after reaction with EDC, sulfo-NHS was added with EDC to form a more stable sulfo-NHS-ester intermediate (Figure 3C)[20]. Immediately following formation of the sulfo-NHS-ester intermediate, primary amine-containing fluorescent dye was added to label the amine-reactive intermediate. In order to minimize microbead aggregation due to amine-reactive sulfo-NHS intermediates binding available primary amine sites on proteins and the microbead surface, carbodiimide crosslinking and fluorescent labeling were performed with intense agitation.

The following parameters were varied to determine their impact on total protein labeling: EDC concentration, sulfo-NHS concentration, crosslinking (EDC + sulfo-NHS) duration, amine-reactive fluorescent dye type and concentration, and dye incubation duration. For each condition, biotinylated rabbit serum attached to microbeads was used without antibody labeling. The operating range and DOR for total protein labeling were determined for each parameter.

Baseline values for EDC concentration (1mM), sulfo-NHS concentration (2.5mM), and crosslinking duration (15 minutes) were selected based on previous studies [20]. We tested EDC concentrations of 10 mM, 1mM, 0.1mM, and 0.01mM. 10mM EDC resulted in substantial

498 microbead aggregation and was thus not further considered (data not shown). We tested sulfo-
499 NHS concentrations of 25mM, 2.5mM and 0.25mM, in conjunction with the preferred EDC
500 concentration. We then tested crosslinking durations of 15 and 30 minutes, using the preferred
501 sulfo-NHS and EDC concentrations. We reasoned that any primary amine-containing dye
502 should be compatible with carbodiimide crosslinking and thus Alexa Fluor 488 hydrazide was
503 arbitrarily chosen as the first dye to be tested, at a concentration of 5µg/mL, based on the
504 manufacturer's suggestions. For cost reasons, higher concentrations of the Alexa Fluor 488
505 hydrazide were not tested. Instead, 4'-(aminomethyl) fluorescein (Thermo-Fisher, Waltham, MA,
506 USA, Supplementary Figure 6), an alternative primary amine-containing fluorescent dye, was
507 tested at concentrations of 5 µg/mL, 50 µg/mL and 500 µg/mL. The preferred dye type and
508 concentration were then tested for incubation times of 30 minutes, 1 hour, 2 hours, and 4 hours.

509 *FRANC Assay Analytical Sensitivity*

510 To determine the analytical sensitivity of the FRANC assay, recombinant proteins of
511 known concentrations were used. Human recombinant TNF-α was conjugated to microbeads
512 over a 24-step protein dilution curve, with 2-fold dilutions at each step, and microbeads at each
513 dilution were exposed to an anti-TNF-α fluorescently-conjugated (PE-Cy7) primary antibody
514 (eBioscience, Waltham, MA, USA). Similarly, we tested human recombinant IL-1β, with the
515 exception that a non-fluorescent anti-IL-1β primary antibody (eBioscience, Waltham, MA, USA)
516 was used followed by three T-PBS wash steps and fluorescent (PE) secondary antibody
517 labeling (Abcam, Cambridge, United Kingdom). Operating regions were determined as above,
518 and the smallest protein amount within the operating region for which the fluorescent signal was
519 greater than the signal from vehicle-only controls was identified as the FRANC assay sensitivity.
520 Three technical replicates were carried out for each recombinant protein type.

Comparing total protein labeling for different protein samples

We next sought to determine how consistent total protein labeling (quantification) was between different samples. Quantification of total protein signal is inherently challenging due to variation in amino acid composition across different proteins. If the labeling chemistry is highly sensitive to amino acid structure, different proteins will be “weighted” differently during quantification, resulting in differences between protein sample types. As a comparison for the FRANC assay, total protein labeling results were compared to those from the widely-used bicinchoninic acid (BCA) assay. However, even the BCA assay is known to give results that vary between protein sample types[22]. Thus, the goal for the FRANC assay’s total protein quantification was to demonstrate inter-sample variability similar (or less than) to the BCA assay.

Goat, rabbit, and mouse sera, as well as cell lysates (mesenchymal stem cells, HT-1080 cells treated with PMA, and HT-1080 untreated cells) were biotinylated, conjugated to microbeads, and then diluted into 96 well plates (2-fold serial dilution over 11 steps). Total protein labeling was performed, followed by flow cytometric analysis. The operating regions (R^2 > 0.90) from plots of total protein fluorescent labeling vs. loaded protein amount were determined for each sample type, as described above. Ideally, if all proteins samples were measured identically by the total protein labeling, the resulting slopes for each sample’s operating range would be equal, whereas any deviation in slope values would suggest that the amino acid composition of the assayed proteins influenced the total protein labeling intensity.

For comparison, the same methodology was utilized with the BCA assay. Each protein sample was prepared for the BCA assay and sample absorbance was measured using a microplate reader (Bio-Tek Synergy H4, Winooski, VT, USA). Results were analyzed similar to total protein labeling, as described above, for each sample. However, the traditional methodology

for determining the BCA assay operating range uses linear regression without the log-log transformations used when analyzing the FRANC assay. To ensure a balanced comparison, BCA and FRANC assay results were analyzed using both methods. The BCA reagent absorbance was plotted vs. standardized sample concentration, and the slope of the linear region was determined. The variability of slope values for the BCA assay was compared to the variability of slope values from the FRANC assay's total protein labeling to determine the FRANC assay's performance.

FRANC Assay Normalization Capabilities

Finally, the normalization capabilities of the FRANC assay were tested using rabbit serum as the protein sample. Biotinylated rabbit serum over a range of concentrations was conjugated to microbeads, and antibody labeling was performed for each microbead sample, followed by total protein labeling. Antibody labelling and total protein labelling values over the concentration range were obtained by flow cytometry, and the operating range was determined for antibody signal vs. loaded sample protein amount. We then normalized the antibody-specific protein signal by the total protein signal across the operating range. The slope of the log-log transformed antibody signal vs. loaded sample protein amount was calculated with and without total protein normalization to determine whether normalization reduced the experimental variability, since a slope closer to 0 implies that the antibody signal is less sensitive to the loaded sample amount. Further, antibody signal across the operating range was normalized relative to actual loaded protein amount, as determined separately by the BCA assay, to compare FRANC assay performance to BCA normalization.

Statistics

To evaluate treatment effects between 3 or more experimental groups, ANOVA was used. The normality of distributions, required for use of ANOVA, was assessed using the Shapiro-Wilk test, with a rejection threshold of $p < 0.05$. To assess whether variances were

equal between groups, also required for ANOVA, we used the Brown-Forsythe test, with a rejection threshold of $p < 0.05$. When ANOVA was suitable, Tukey's post hoc test was used to compare all groups or Dunnett's post hoc test was used to compare each group mean to a single control mean, where $p < 0.05$ indicated significant differences.

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Author Contributions

ES prepared the main manuscript text, developed the assay idea, performed experiments, and interpreted data. JR assisted with the development of the assay idea, performed experiments and interpreted data. AC and KK assisted with experiments, manuscript review and data analysis. CE provided financial support and feedback on the manuscript, experiment design, and data interpretation.

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